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ORIGINAL ARTICLE

Neuroprotective effects of AT1 receptor antagonists after experimental ischemic stroke: what is important?

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Abstract The present study conducted in rats defines the requirements for neuroprotective effects of systemically administered AT1 receptor blockers (ARBs) in acute ischaemic stroke. The inhibition of central effects to angiotensin II (ANG II) after intravenous (i.v.) treatment with candesartan (0.3 and 3 mg/kg) or irbesartan and losartan (3 and 30 mg/kg) was employed to study the penetration of these ARBs across the blood-brain barrier. Verapamil and probenecid were used to assess the role of the transporters, P-glycoprotein and the multidrug resistance-related protein 2, in the entry of losartan and irbesartan into the brain. Neuroprotective effects of i.v. treatment with the ARBs were investigated after transient middle cerebral artery occlusion (MCAO) for 90 min. The treatment with the ARBs was initiated 3 h after the onset of MCAO and continued for two consecutive days. Blood pressure was continuously recorded before and during MCAO until 5.5 h after the onset of reperfusion. The higher dose of candesartan completely abolished, and the lower dose of candesartan and higher doses of irbesartan and losartan partially inhibited the drinking response to intracerebroventricular ANG II. Only 0.3 mg/kg candesartan improved the recovery from ischaemic stroke, and 3 mg/kg candesartan did not exert neuroprotective effects due to marked blood pressure reduction during reperfusion. Both doses of irbesartan and losartan had not any effect on the stroke outcome. An effective, long-lasting blockade of brain AT1 receptors after systemic treatment with ARBs without extensive blood pressure reductions is the prerequisite for neuroprotective effects in ischaemic stroke.

Keywords Angiotensin II · AT$_1$ receptor antagonists · Blood-brain barrier · Cerebral ischaemia · Neuroprotection

Introduction

Angiotensin II (ANG II) plays an important role in the pathogenesis of stroke. An exclusive blockade of brain AT1 receptors without affecting AT1 receptors in the periphery was demonstrated to exert neuroprotective effects (Dai et al. 1999; Yamakawa et al. 2003; Lou et al. 2004). This has been convincingly demonstrated, as intracerebroventricular (i.c.v.) infusion of the AT1 receptor blocker (ARB), irbesartan prior and during transient middle cerebral artery occlusion (MCAO) improved the recovery from cerebral ischaemia (Dai et al. 1999; Yamakawa et al. 2003; Lou et al. 2004; Li et al. 2005). A long-term systemic treatment with ARBs prior to stroke exerts beneficial effects on blood vessel morphology in hypertensive rats, normalises cerebrovascular autoregulation, inhibits the penetration of inflammatory cells into the brain parenchyma and promotes angiogenesis (Ito et al. 2002; Groth et al. 2003; Ando et al. 2004; Forder et al. 2005). Pre-treatment with ARBs is, however, not required for neuroprotection, as the results of a number of experimental studies have shown beneficial effects of ARBs administered after the completion of the ischaemic insult (Sugawara et al. 2005; Liu et al. 2008; Engelhorn et al. 2004; Fagan et al. 2006; Brdon et al. 2007). Collectively, the results of experimental studies in rodent models of stroke suggest that ARBs are effective in reducing infarct volume and improving neurological

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outcome regarding dose timing or ischaemic model. Surprisingly, the results of two clinical trials, the ACESS and SCAST trial, investigating the effects of candesartan treatment after acute ischaemic stroke were disappointing, and moreover, the results of the SCAST trial even suggested possible harmful effects (Schrader et al. 2003; Sandset et al. 2011). The questions arise: Firstly, why did the bench to bedside translation in this particular case failed? Secondly, under which conditions that the systemic treatment with ARBs after ischaemic stroke can be neuroprotective? The aim of this study was to determine the conditions upon which treatment with ARBs in the post-ischaemic phase confers neuroprotection and improves the recovery from stroke. We investigated the effects of systemic treatment with three ARBs, candesartan, losartan and irbesartan, on infarct volume, oedema formation and neurological outcome in rats exposed to MCAO and made an attempt to correlate the effects on the functional status after stroke with their ability to cross the blood-brain barrier (BBB). Furthermore, we also assessed the role of the transporters, P-glycoprotein (P-gp) and the multidrug resistance protein-2 (MRP-2) in the entry of candesartan, losartan and irbesartan into the brain. Two doses of each ARB were employed in the study, a low dose, which does not or only marginally reduces blood pressure after MCAO, and a dose which promotes a profound decrease in blood pressure in the post-stroke phase.

Materials and methods

Animals

Male, normotensive Wistar rats weighing 200–220 g were obtained from Charles River (Sulzfeld, Germany). Animals were kept under controlled conditions with respect to temperature and humidity and were housed on a 12-h light/12-h dark cycle with free access to food and water.

Treatments and experimental design

All experimental protocols were approved by the Governmental Committee for the Ethical Use of Experimental Animals in the German Federal State of Schleswig-Holstein. All efforts have been made to minimise animal suffering and to reduce the number of animals used in our experiments. In all experiments, drug allocation was randomised throughout the respective study period.

Experiment 1

The penetration of systemically administered ARBs through the BBB was investigated by their ability to block the brain-mediated effects of i.c.v. administered ANG II such as the drinking response. Rats were treated s.c. with candesartan (0.3 mg/kg, $n = 9$ and 3 mg/kg, $n = 9$), irbesartan (3 mg/kg, $n = 12$ and 30 mg/kg, $n = 11$) and losartan (3 mg/kg, $n = 11$ and 30 mg/kg, $n = 9$). Vehicle-treated animals (0.1 N Na_2CO_3) ($n = 11$) served as controls. The water intake in response to ANG II-injected i.c.v. (100 ng) was measured 1 day before and 2 h after administration of the antagonists.

Experiment 2

The neuroprotective effects of these antagonists were investigated in rats subjected to MCAO lasting for 90 min followed by reperfusion. The onset of treatment was 3 h after the onset of focal brain ischaemia, i.e. 90 min after the onset of reperfusion. Rats were treated s.c. with candesartan (0.3 and 3 mg/kg), irbesartan (3 and 30 mg/kg), losartan (3 and 30 mg/kg) or vehicle (0.1 N Na_2CO_3) controls. Treatment with vehicle and the AT1 receptor antagonists was continued for two consecutive days after MCAO ($n = 15$ per treatment group). The evaluations of neurological outcome were carried out 24 and 48 h after the onset of reperfusion. Two days after focal cerebral ischaemia, animals were perfused intracardially with 4% paraformaldehyde under deep anaesthesia. The brains were removed and used for the measurement of infarct volume and oedema volume.

Experiment 3

Six rats of each group were used for the measurement of blood pressure. Rats were treated exactly as in experiment 2, and the mean arterial blood pressure (MAP) was recorded before and during MCAO and up to 4 h after drug application.

Experiment 4

The modulation of the penetration of ARBs through the BBB by transporters, P-gp or MRP-2, was studied using verapamil and probenecid as the transporter inhibitors. ANG II (100 ng) was injected i.c.v., and the water intake was measured over a time period of 20 min. The drinking response was determined before and 0.5 and 4 h after i.v. administration of vehicle ($n = 37$), losartan (10 mg/kg) ($n = 12$), irbesartan (30 mg/kg) ($n = 12$) and candesartan (0.33 mg/kg) ($n = 19$). After a washout period of 2 days, the P-gp inhibitor, verapamil (10 mg/kg), or the MRP-2 inhibitor, probenecid (90 mg/kg), was injected i.p. 1 h before administration of the ARBs and the drinking experiments were repeated.

Anaesthesia

Chloral hydrate (400 mg/kg body weight) injected intraperitoneally was used for all surgical procedures.

Implantation of the femoral artery catheter

A polyethylene catheter (PP-50) for measurements of MAP was inserted through the femoral artery into the abdominal aorta 2 days before MCAO. The catheter was filled with heparinised saline, passed through a subcutaneous tunnel and sealed at the back of the neck.

Middle cerebral artery occlusion with reperfusion

In this study, the intraluminal occlusion of the MCA for 90 min with subsequent reperfusion was used (Koizumi et al. 1986). Regional cerebral blood flow (rCBF) was continuously monitored in each rat at one point (1 mm posterior to the bregma, 5 mm from the midline) on the surface of each hemisphere in the supply territory of the MCA before during and after MCAO by laser-Doppler-flowmetry (Periflux system 5000) (Schmid-Elsaesser et al. 1998). The measurements of rCBF were carried out to assess the degree of blood flow reduction. Abrupt reduction in rCBF by approximately 75 to 90% indicated a successful occlusion of the MCA. Rats in which the ipsilateral blood flow during ischaemia was not reduced to less than 25% of baseline during the first 30 min of occlusion, or in which a premature increase in the ipsilateral blood flow was recorded, as well as rats with subarachnoid haemorrhage were excluded from the protocol. Body temperature was maintained at 37 °C with a heating pad.

Blood pressure measurement

Measurements of MAP were carried out via the arterial catheters as described previously (Culman et al. 1997). The experiments were started when the animals were resting and when the basal MAP and heart rate were stable. MAP was continuously measured starting 1 h before the induction of anaesthesia, during MCAO and up to 4 h after application of vehicle or the ARBs.

Neurological deficits

Neurological evaluations were carried out daily by a blinded observer 24 and 48 h after the surgery. Two neurological evaluation grading systems were used. The neurological evaluation developed by Bederson et al. (1986) (grading scale 0–3) includes the assessment of the grade of the forelimb flexion contralateral to the injured hemisphere, resistance to lateral push and observation of circling behaviour. Rats with no observable neurological deficits were graded 0. Rats with forelimb flexion and shoulder adduction were graded 1. Grade 2 was assigned to severely dysfunctional rats with decreased resistance to lateral push towards the paretic side. Rats displaying consistent circling behaviour together with forelimb flexion and decreased resistance to lateral push were graded 3 (Bederson et al. 1986). The neurological evaluation described by Garcia et al. (1995) consists of the following six tests: (1) spontaneous activity, (2) symmetry in the movement of four limbs, (3) forepaw outstretching, (4) climbing, (5) body proprioception and (6) response to vibrissae touch. Severe impairments in each of the tests were graded 0 or 1, while no observable deficits were graded 3. The minimum neurological score is 3, and the maximum neurological score, e.g. intact rats, is 18.

Tissue processing

Two days after MCAO, rats were deeply anaesthetised and intracardially perfused with phosphate-buffered saline (pH 7.4) followed by 4% paraformaldehyde. The brains were removed, post fixed overnight and subsequently cryoprotected in 30% sucrose for 72 h at 4 °C. Coronal sections (40 μm) were cut in a cryostat from the level bregma +3.7 mm to the level bregma −6.7 mm. Every 20th slice was used for the determination of the infarct size and oedema volume.

Measurement of infarct volume and oedema volume

To measure the infarct area, 14 coronal brain sections from different levels of the brain (see above) were stained with cresyl violet. Slice images were digitalised, and the area of infarct was determined on each slice (Leica QWin image analysis system). The infarct volume was calculated by multiplying the sum of the infarct areas with the distance between the sections. The volumes of the ipsilateral and contralateral hemispheres were measured, and the difference between both areas in each section was used to calculate the oedema volume. The correction of the infarct size for oedema was calculated using the formula of Swanson et al. (1990).

Statistical analyses

All values are expressed as the means ± SEM. Comparisons of neurological outcomes and MAP were analysed by two-way analysis of variance followed by a post hoc Tukey test for pairwise comparisons at individual time points. Comparisons of the drinking responses to i.c.v. ANG II and the infarct and oedema volumes were carried out by ANOVA followed by post hoc Tukey test. Statistical significance was accepted at $p < 0.05$.
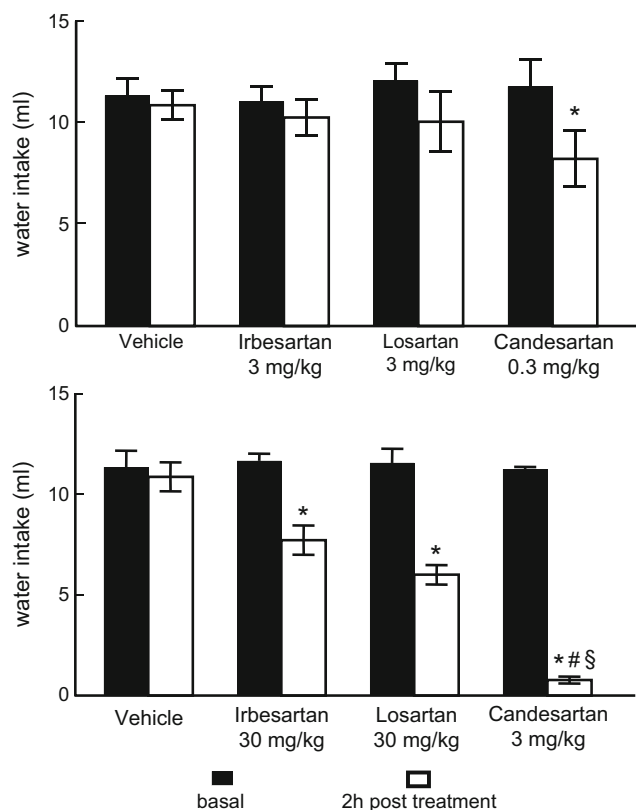
Results

Drinking response to ANG II

The drinking response to i.c.v. ANG II was partly inhibited after systemic treatment with the low dose of candesartan, but

not with the low doses of losartan or irbesartan. Treatment with the high dose of candesartan completely abolished the drinking response to i.c.v. ANG II, while the high doses of losartan and irbesartan only partially attenuated the response (Fig. 1).

Neurological outcome after focal cerebral ischaemia

The rCBF in the supply territory of the middle cerebral artery decreases by 25–30% in sham-operated rats. Previous findings have convincingly demonstrated no significant differences in neurological scores of motor deficits and sensory impairments between sham-operated rats and intact controls at any time point after the sham-operation (Culman et al. 2012). On the contrary, reductions in the rCBF by 75–90% during MCAO induce severe neurological deficits regardless of the treatment. To assess the effects of ARBs on the recovery from ischaemic stroke, we merely compared the neurological outcomes of post-ischaemic treatment with various ARBs to the neurological status of vehicle-treated rats subjected to cerebral ischaemia; sham-operated animals were not included. Rats treated subcutaneously with 0.3 or 3 mg/kg candesartan
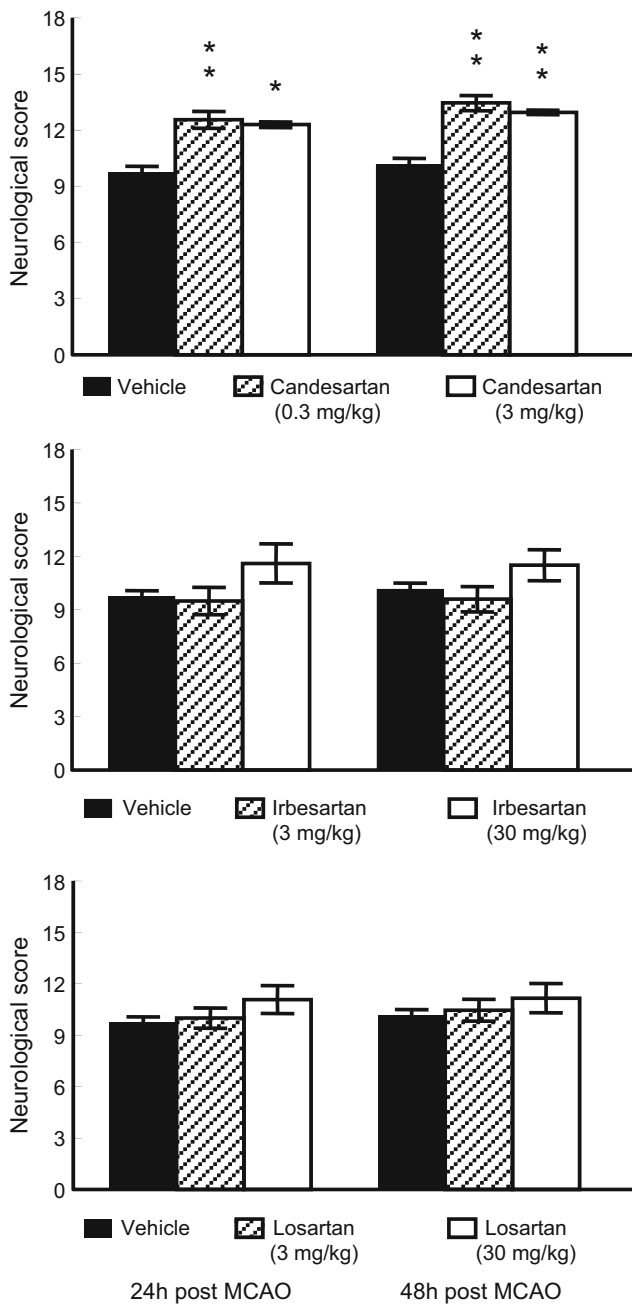
showed a significantly improved neurological outcome compared with rats treated with vehicle. Both irbesartan and losartan at either dose did not affect the neurological outcome (Fig. 2). Similar results were obtained when the neurological examination developed by Garcia et al. (1995) was employed (Fig. 3).
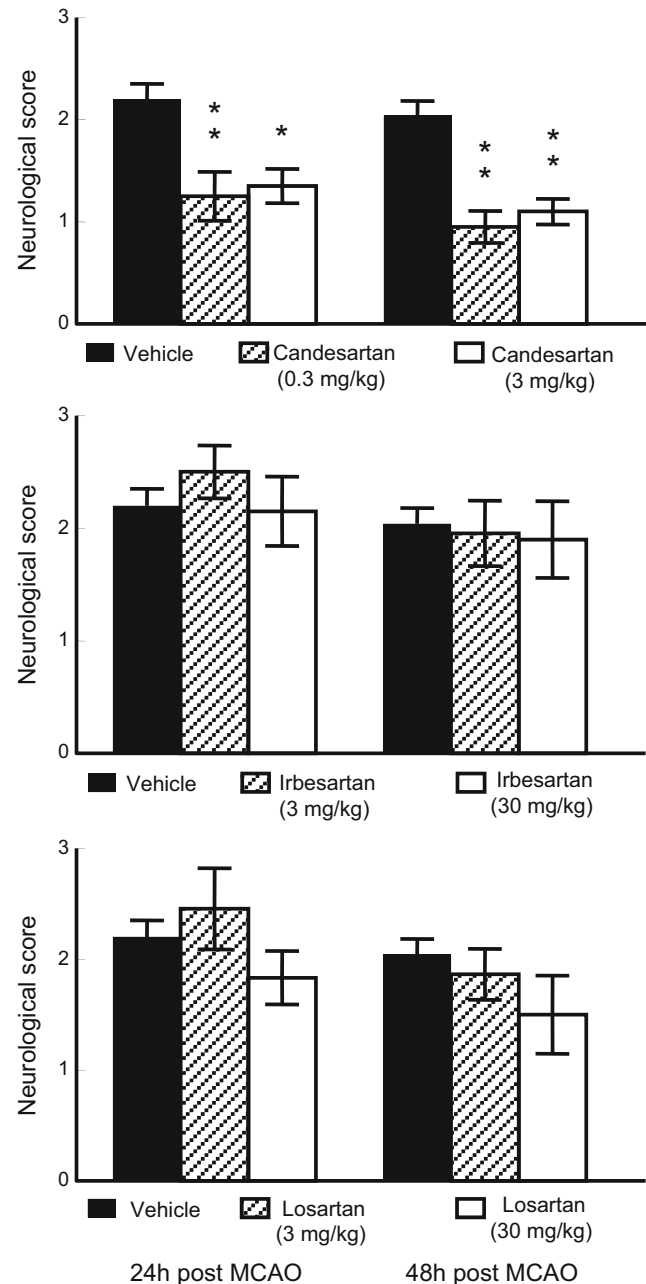
Fig. 2 Neurological scores in normotensive rats treated systemically with vehicle, candesartan (0.3 and 3 mg/kg s.c.), irbesartan (3 and 30 mg/kg s.c.) and losartan (3 and 30 mg/kg s.c.). Drug treatment was started 3 h after the initiation of middle cerebral artery occlusion (MCAO). The neurological evaluation grading system 0–3 developed by Bederson et al. (14) was used. Please notice that rats displaying better neurological outcome receive lower neurological score. Data are expressed as the means ± SEM. *$p < 0.05$; **$p < 0.01$ vs vehicle-treated animals

Fig. 1 Effect of systemic treatment with vehicle, candesartan (0.3 and 3 mg/kg s.c.), irbesartan (3 and 30 mg/kg s.c.) and losartan (3 and 30 mg/kg s.c.) on the drinking response induced by ANG II (100 ng) injected i.c.v. before (basal) and 2 h post-antagonist treatment. *$P < 0.05$ vs vehicle; #$P < 0.05$ vs irbesartan (30 mg/kg); §$P < 0.05$ vs losartan (30 mg/kg)

Fig. 3 Neurological scores in normotensive rats treated systemically with vehicle, candesartan (0.3 and 3 mg/kg s.c.), irbesartan (3 and 30 mg/kg s.c.) and losartan (3 and 30 mg/kg s.c.). Drug treatment was started 3 h after the initiation of middle cerebral artery occlusion (MCAO). The neurological evaluation grading system 3–18 developed by Garcia et al. (15) was used. Rats with better neurological outcome receive higher neurological score. Data are expressed as the means ± SEM. *$p < 0.05$; **$p < 0.01$ vs vehicle-treated animals
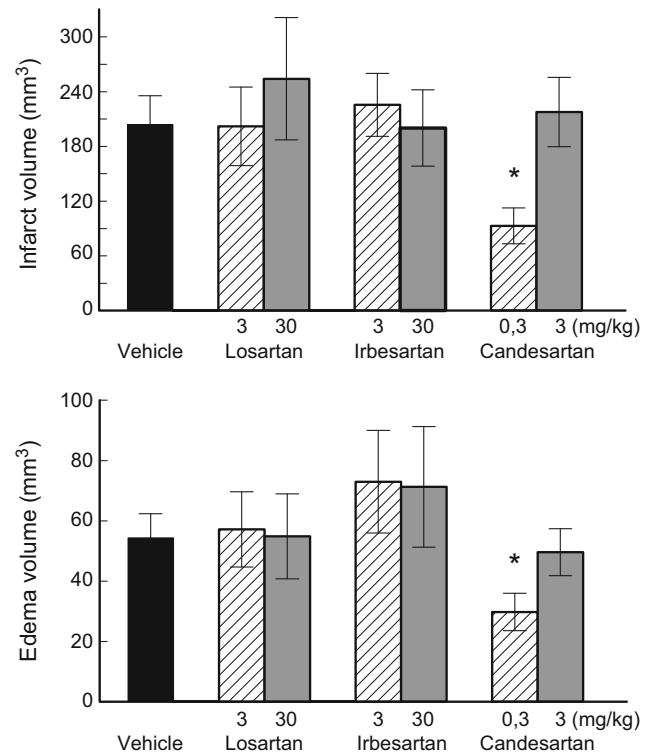
Infarct and oedema volumes

The size of the ischaemic injury was determined by cresyl violet staining of brain slices 2 days after MCAO with reperfusion. The cytoarchitecture of the cortex was normal in the contralateral hemisphere. Two days after focal cerebral ischaemia, severe unilateral injury was clearly detected as area of pallor which was sharply demarcated from the adjacent tissue. The ischaemic lesion covered the supply territory of the middle cerebral artery comprising parts of the frontoparietal and frontotemporal cortex and the lateral and medial segments of the caudate putamen.

Low dose of candesartan significantly reduced infarct and oedema volumes compared to the vehicle-treated group by 54 and 45%, respectively (Fig. 4). In contrast, either dose of losartan and irbesartan as well as the high dose of candesartan had not any effect on both parameters (Fig. 4). Preliminary results obtained in our laboratory have shown that a moderate rCBF reduction in sham-operated rats is not sufficient to induce ischaemic neuronal death. Consequently, no infarction and oedema could be detected in these rats (data not shown).

Mean arterial blood pressure

In all rats, blood pressure decreased following anaesthesia and gradually increased during MCAO and reperfusion. Treatment with the high doses of all AT1 receptor antagonists starting 3 h after the onset of MCAO significantly reduced
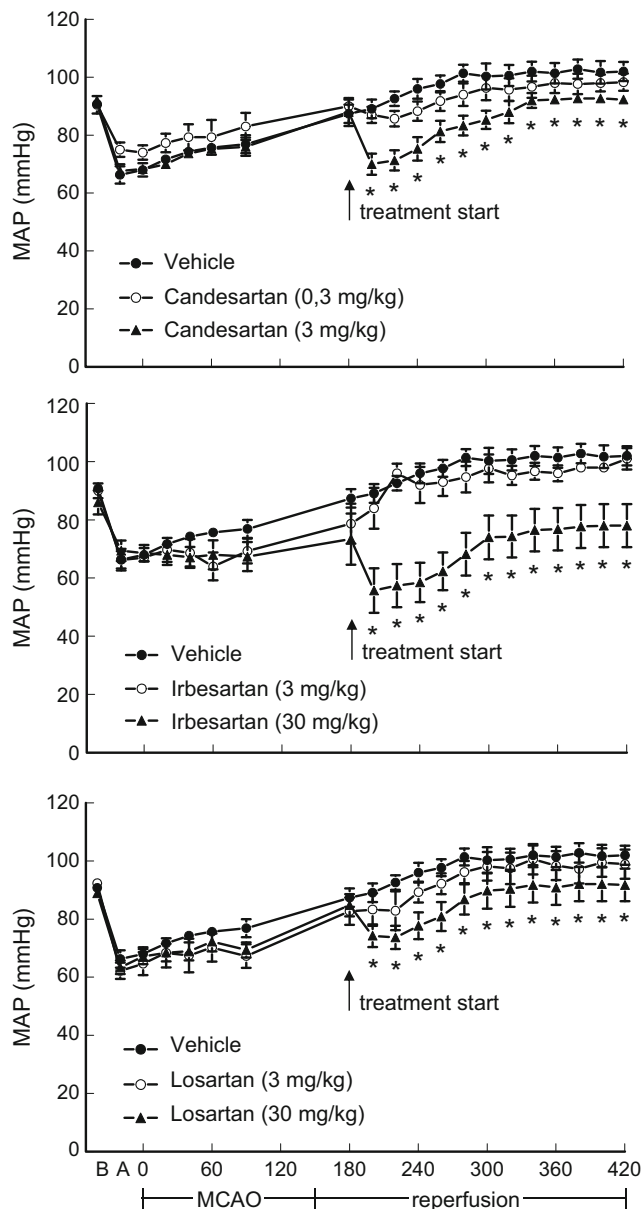

Fig. 4 Effect of systemic treatment with vehicle, candesartan (0.3 and 3 mg/kg s.c.), irbesartan (3 and 30 mg/kg s.c.) and losartan (3 and 30 mg/kg s.c.) on the total infarct volume (*upper panel*) and on oedema volume (*lower panel*) 2 days after MCAO for 90 min. Drug treatment was started 3 h after the initiation of middle cerebral artery occlusion. Data are expressed as the means ± SEM. *$p < 0.05$ vs vehicle-treated animals

blood pressure compared to vehicle. Blood pressure was not significantly altered after treatment with the low doses of the antagonists (Fig. 5).

Blockade of P-gp and MRP-2

Losartan, irbesartan and candesartan were administered i.v. at doses which block the drinking response to i.c.v. ANG II by

Fig. 5 Mean arterial blood pressure (MAP) at baseline (B) after anaesthesia (A) during occlusion of the middle cerebral artery for 90 min (MCAO) and during the reperfusion period in rats treated systemically with vehicle, candesartan (0.3 and 3 mg/kg s.c.), irbesartan (3 and 30 mg/kg s.c.) and losartan (3 and 30 mg/kg s.c.). Drug treatment was started 3 h after the onset of MCAO. Data are expressed as the means ± SEM. *$p < 0.05$ statistical comparisons with the vehicle treated group at the given time point

about 30–50% 0.5 h post-treatment. The drinking response to i.c.v. ANG II was not affected by i.v. treatment with vehicle alone or following pre-treatment with verapamil or probenecid (Figs. 6a and 7a). The inhibition of the drinking response to i.c.v. ANG II was further enhanced by candesartan and losartan after 4 h (Figs. 6b, c and 7b, c), but was nearly absent following administration of irbesartan (Figs. 6d and 7d). Pre-treatment with verapamil or probenecid had no effect on the inhibitory action of losartan and irbesartan (Figs. 6c, d and 7c, d). Pre-treatment of rats with verapamil significantly enhanced the inhibitory action of candesartan on the drinking response to i.c.v. ANG II (Fig. 6b), while pre-treatment with probenecid was without effect (Fig. 7b).

Discussion

The results of the present study, clearly defining the requirements for the neuroprotective effects of ARBs after ischaemic stroke, may help to explain why the clinical trials, ACCESS and especially SCAST have produced negative results.

Firstly, an effective inhibition of brain AT1 receptors plays a crucial role for the neuroprotective actions of ARBs after acute ischaemic stroke. Long-lasting blockade of brain AT1 receptors achieved by a long-term i.c.v. infusion of irbesartan reduced the infarct size, inflammation and apoptosis and improved the neurological outcome of focal cerebral ischaemia (Dai et al. 1999; Lou et al. 2004). Certainly, an efficient inhibition of AT1 receptors in the penumbra considerably contributes to the reduced inflammation in the peri-infarct areas and prevention of infarct growth and is, therefore, decisive for the improved recovery from ischaemic stroke (Dirnagl et al. 1999; Yanagitami et al. 1999; Neher et al. 2002). However, MCAO also initiates inflammation, oxidative damage and loss of neurons in areas remote from the site of ischaemic injury, such as the hippocampus or substantia nigra (Morioka et al. 1993; Volpe et al. 1995; Huh et al. 2003). Therefore, we propose that the penetration of ARBs across the BBB and effective inhibition of AT1 receptors not only in the ischaemic core and penumbra but also in brain areas not directly affected by the ischaemic insult are decisive for their neuroprotective actions.

Secondly, an effective and, even more importantly, long-lasting inhibition of brain AT1 receptors is essential for the reduction of ischaemic brain injury. A number of studies have reported that candesartan inhibits brain AT1 receptors and, consequently, the central effects of ANG II more effectively than other ARBs, such as losartan, irbesartan or telmisartan. Indeed, even high doses of systemically administered losartan or irbesartan failed to completely abolish the central ANG II effects and the moderate inhibition was only short-lasting (Polidori et al. 1996; Polidori et al. 1998; Culman et al. 1999; Gohlke et al. 2001; Gohlke et al. 2002a, 2002b). The
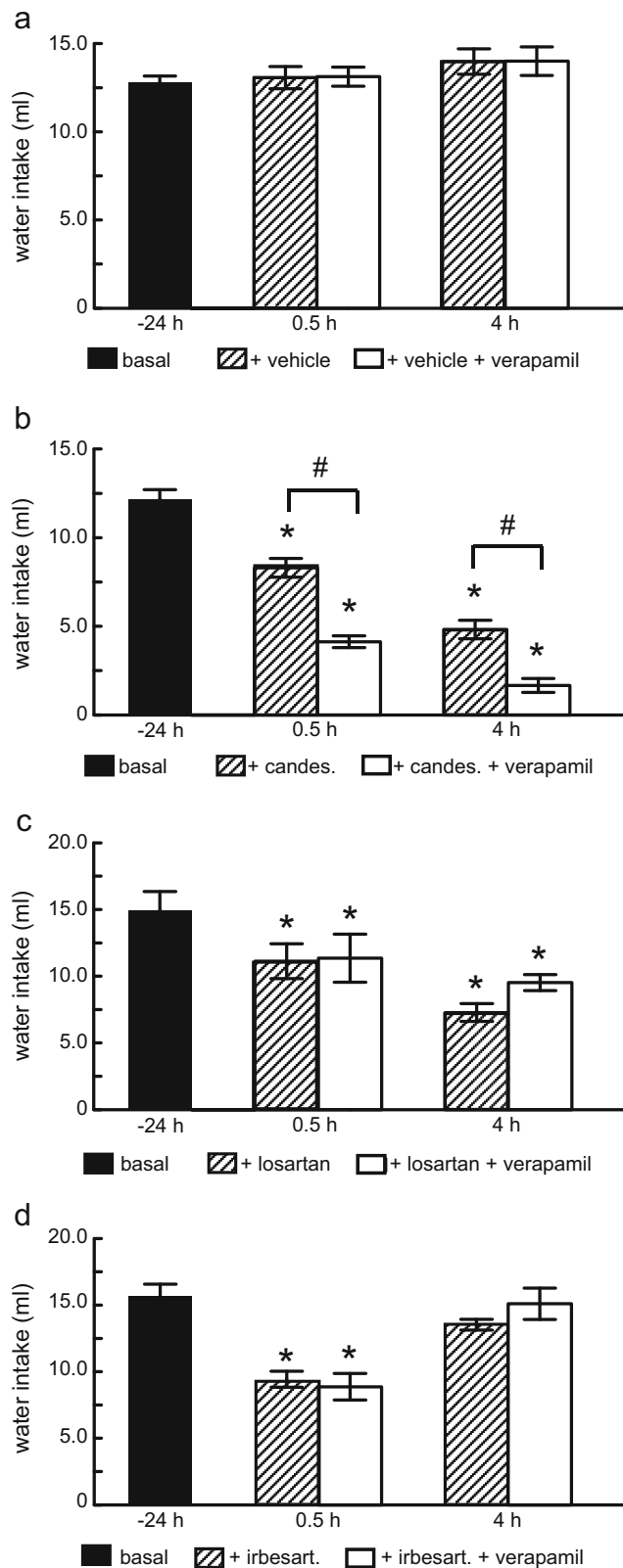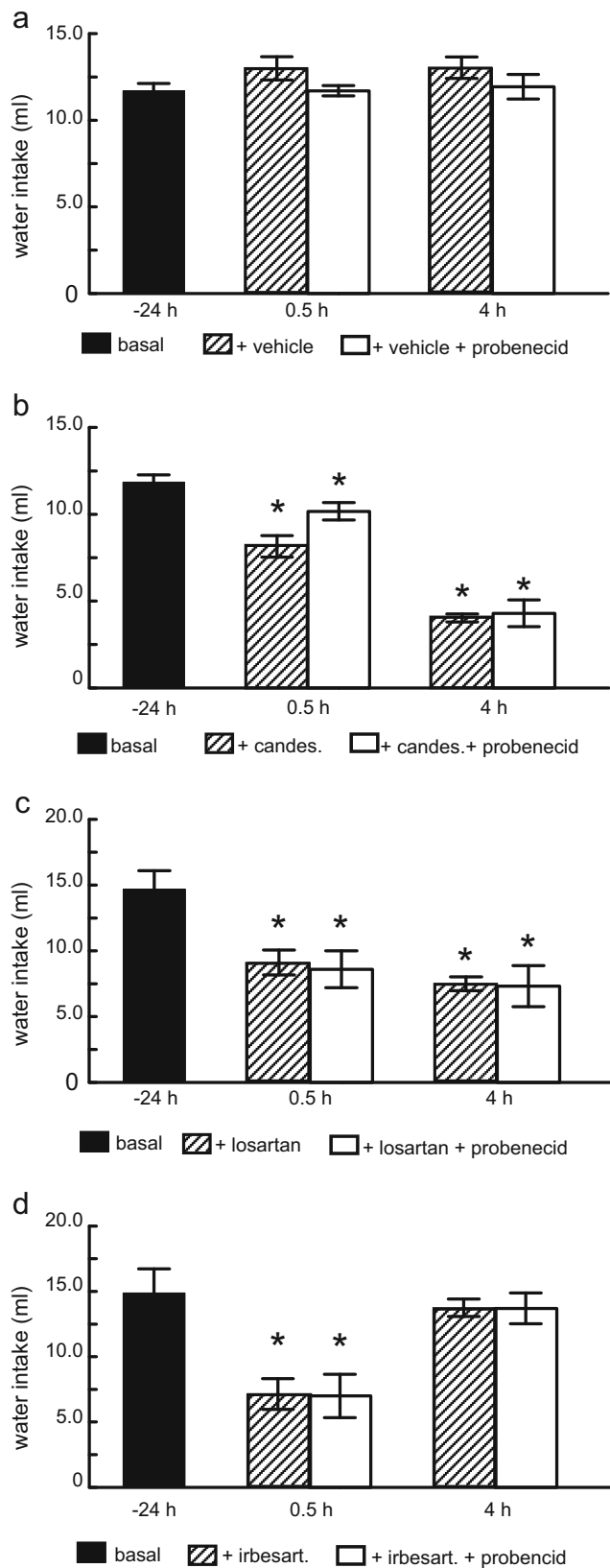
Fig. 6 **a**–**d** Drinking response to angiotensin II (i.c.v.), 24 h prior and 0.5 and 4 h post i.v. injection of vehicle (**a**), candesartan (0.33 mg/kg) (**b**), losartan (10 mg/kg) (**c**) or irbesartan (30 mg/kg) (**d**) in the presence or absence of the P-gp inhibitor, verapamil. Verapamil (10 mg/kg) was administered i.p. 1 h before vehicle, candesartan, losartan or irbesartan injection. Data are expressed as the means ± SEM. $*p < 0.05$ vs basal; $^\#p < 0.05$ vs candesartan alone

and irbesartan. The superior inhibitory effect of candesartan on brain AT1 receptors cannot be simply explained by a better passive penetration of the antagonist through the BBB. Losartan and irbesartan are certainly more lipophilic than candesartan (Morsing et al. 1999). Along with tight junctions between cerebral endothelial cells constituting a physical barrier between the brain and blood, numerous membrane efflux transporters, such as P-gp, the MRPs 1–6 and Breast Cancer Resistance Protein (BRCP), in cerebral endothelial cells prevent the penetration of substances into the brain parenchyma (Abbott et al. 2010; Leslie et al. 2005; Potschka et al. 2003). In vitro studies have revealed that losartan, but not irbesartan and EXP-3174, is a substrate for P-gp (Soldner et al. 2000). We demonstrate here that the blockade of P-gp by verapamil or MRP-2 by probenecid did not alter the efficacy of losartan and irbesartan to inhibit the ANG II-mediated effects in the brain. It is worthy to state that besides P-gp and MRP-2, verapamil and probenecid also inhibit other transporters (Sugiyama et al. 2001; Löscher and Potschka 2005; Chen et al. 2014). Therefore, the failure of systemically administered losartan and irbesartan to inhibit central effects of ANG II as effectively as candesartan cannot be simply explained by their outward transport via efflux transporters. Verapamil further increased the candesartan-induced inhibition of the drinking response to i.c.v. ANG II, indicating that P-gp can obviously limit the penetration of candesartan through the BBB. These findings are somewhat surprising and unexpected and do not explain the differences in the penetration of the three ARBs through the BBB and, consequently, their different potencies to attenuate the drinking response to Ang II.

Moreover, the dissociation rate of these antagonists from the antagonist-receptor complex, which in turn correlates with the degree of insurmountable inhibition, also plays a role in the neuroprotective actions of ARBs (Vanderheyden et al. 2000; Van Liefde and Vauquelin 2009). The much slower dissociation of candesartan, compared to irbesartan and losartan, from the AT1 receptor can explain why only candesartan (the lower dose) significantly reduced the infarct size and oedema. Collectively, effective penetration of the ARBs into the brain parenchyma and the high degree of the insurmountable antagonism are essential for the reduction of ischaemic brain injury.

Thirdly, ARBs exert beneficial effects only when excessive blood pressure reduction in the post-stroke phase is avoided. Blood pressure, which is usually increased in the post-stroke

present data is concurrent with this assumption. The lower dose of candesartan attenuated the central effects of ANG II to a greater extent and for a longer period of time than losartan

◀ **Fig. 7 a–d** Drinking response to angiotensin II (i.c.v.), 24 h prior and 0.5 and 4 h post i.v. injection of vehicle (**a**), candesartan (0.33 mg/kg) (**b**), losartan (10 mg/kg) (**c**) or irbesartan (30 mg/kg) (**d**) in the presence or absence of the MRP-2 inhibitor, probenecid. Probenecid (90 mg/kg) was administered i.p. 1 h before vehicle, candesartan, losartan or irbesartan injection. Data are expressed as the means ± SEM. $*p < 0.05$ vs basal

and it becomes dependent on the systemic blood pressure. Reduction of blood pressure reduces blood flow to ischaemic penumbra and worsens the tissue damage. Extreme arterial hypertension is clearly detrimental, but moderate arterial hypertension may be advantageous by improving cerebral perfusion of the ischaemic tissue. Decreases in blood pressure in the acute post-stroke phase were associated with poor clinical outcomes (Jauch et al. 2013).

Our present data is consistent with earlier observations (Engelhorn et al. 2004; Fagan et al. 2006; Brdon et al. 2007). The low dose of candesartan (0.3 mg/kg), which did inhibit brain AT1 receptors but did not lower blood pressure in the post-stroke phase, improved the recovery from ischaemic stroke. In contrast, the higher dose of candesartan (3 mg/kg), which completely abolished the central effect of ANG II, failed to reduce the infarct size, presumably because of pronounced blood pressure decreases in the reperfusion period. Obviously, the excessive blood pressure reduction overrode the neuroprotective actions of candesartan in the brain. Losartan and irbesartan did not exert any neuroprotection because low doses of these ARBs failed to effectively interact with brain AT1 receptors, while higher doses markedly reduced blood pressure after ischaemic insult. Our data convincingly demonstrates that besides effective inhibition of brain AT1 receptors, ARBs exert beneficial effects only when the moderately increased arterial blood pressure after the ischaemic insult is not significantly lowered. Although these results were obtained in rats, in which anaesthesia lowered blood pressure during MCAO, similar conclusions were also drawn when rats were shortly anaesthetised only for the induction of cerebral ischaemia. Under these experimental conditions, blood pressure increased rapidly during MCAO and slowly declined during the reperfusion period. Candesartan injected i.v. at the beginning of reperfusion reduced infarct volume and improved the neurological score (Fagan et al. 2006).

The conclusions derived from the present study conform to the clinical data. The ACCESS study reports on decreased cardiovascular morbidity and mortality in patients treated with candesartan cilexetil in the early phase of stroke. Candesartan, however, did not affect the functional outcome most likely due to small sample size of patients. The trial had to be stopped prematurely on the recommendation of the safety committee, when only 339 valid patients had been randomised, because of an imbalance in end points (Schrader et al. 2003). In the SCAST trial, blood pressure lowering treatment with candesartan was not beneficial in patients suffering from acute stroke; the

phase, is decisive for the clinical outcome. In the early post-stroke phase, the cerebral blood flow regulation is impaired

evidence even suggested possible harmful effects (Sandset et al. 2011). In both studies, treatment with candesartan cilexetil was started using a low dose of 4 mg of the ARB per day. In the ACCESS study, the dosage was increased to 8 or 16 mg candesartan cilexetil only if blood pressure exceeded 160 mmHg systolic or 100 mmHg diastolic, i.e. in the majority of patients the dose of candesartan remained the same over the whole period of treatment. In the SCAST study, however, there was a fixed dose escalation scheme: 4 mg on day 1, 8 mg on day 2 and 16 mg on days 3–7. Correspondingly, blood pressure values in the ACCESS study were not significantly different between the candesartan- and placebo-treated groups, whereas in the SCAST study blood pressure on day 7 was significantly lower in the candesartan group than in placebo group. Although symptomatic hypotension in the SCAST trial was recorded for 1% patients receiving increasing doses of candesartan, it is conceivable to assume that in a large number of patients, a marked drop of blood pressure within the first hours and days induced by the intense antihypertensive treatment significantly reduced cerebral blood flow to ischaemic areas which, in turn, might have negative effects on the functional outcome.

The timing of the start of treatment appears to be crucial in patients with acute stroke. In a subgroup of patients in the SCAST study, treatment with candesartan was associated with a reduced risk of cardiovascular events only when treatment was started during the first 6 h after the stroke onset (Sandset et al. 2011, Jusufovic et al. 2016). Similar conclusions indicating that only early, but not delayed begin of the treatment after stroke is beneficial, have been drawn from studies employing NO donors. NO donors improved functional outcome in stroke patients and reduced lesion size in experimental models of cerebral ischaemia only when administered early after the stroke onset (Woodhouse et al. 2015, Ankolekar et al. 2013, Willmot et al. 2005). The recent subgroup analysis of the SCAST trial demonstrating a significant trend towards a better functional outcome of candesartan in patients with larger than in patients with smaller, lacunar infarcts, supports the view of beneficial effects of AT1 receptor blockade in the brain in acute stroke (Sandset et al. 2015).

The present findings clearly demonstrate that only candesartan, but not losartan or irbesartan, confers neuroprotection after transient ischaemic stroke at doses which, on one side, effectively block brain AT1 receptors over a longer period of time and, on the other side, have only minor effects on arterial blood pressure. In our experiments, we employed the intraluminal suture MCAO, which is the most frequently used model for experimental stroke in rats and mice. This method allows performing permanent and transient ischaemia in a controlled manner. In contrast to the permanent stroke models, consequences of reperfusion such as reperfusion injury have to be taken into account. Rat models of ischaemic stroke are usually conducted in young animals of the same age lacking chronic diseases or genetic predisposition. In clinical trials,

however, there is a high variability in the cause, duration, localisation and severity of ischaemia. The majority of stroke patients included in clinical trials suffer from ongoing ischaemia. In the SCAST study, for example, only 8% of the candesartan-treated and 9% of the placebo-treated patients received recanalisation by thrombolytic treatment before randomisation. Although the model of the transient ischaemic stroke was used in the present study, we strongly believe that an effective penetration of the ARBs across the BBB and long-lasting inhibition of brain AT1 is essential for the reduction of brain injury and better recovery from both transient and permanent cerebral ischaemia. Furthermore, ARBs exert neuroprotection only when reductions of moderately increased arterial blood pressure in the post-stroke phase are avoided.

Conflict of interest The authors declare that they have no conflict of interest.

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